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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 38/00, C07K 14/705

A1

(11) International Publication Number: WO 98/09638

(43) International Publication Date: 12 March 1998 (12.03.98)

(21) International Application Number: PCT/US97/15586

(22) International Filing Date: 5 September 1997 (05.09.97)

(30) Priority Data:
60/025,846 6 September 1996 (06.09.96) US

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(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: INHIBITION OF MAST CELL ACTIVATION BY gp49-BASED MECHANISMS AND REAGENTS

(57) Abstract

Mouse mast cells express gp49B1, a cell-surface member of the immunoglobulin (Ig) superfamily encoded by the gp49B gene. We now report a human gp46 family. Our findings establish a novel counter-regulatory transmembrane pathway by which mast cell activation can be inhibited.

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05311/017WO1

INHIBITION OF MAST CELL ACTIVATION BY gp49-BASED MECHANISMS AND REAGENTS

Statement as to Federally Sponsored Research

This work was supported in part by grants AI-07306, AI-22531, AI-21101, AI-31599, AI-32101, and HL-36110 from the National Institutes of Heath and by an institutional grant from INSERM/CNRS (EV).

Background of the Invention

The rat monoclonal antibody (mAb)[1] B23.1 recognizes an epitope expressed on an ~49-kDa glycoprotein on the surface of mouse mast cells that is synthesized from an intracellular 37-kDa protein precursor (Katz et al., 1989, J. Immunol. 142:919-926). Immunoaffinity purification of a cell-surface protein from mast cells with mAb B23.1, determination of its amino-terminal amino acid sequence, and screening of a mast cell cDNA library with oligonucleotides encoding the amino-terminal sequence provided three cDNAs that encode this amino-terminal amino acid sequence (Arm et al., 1991, J. Biol. Chem. 266:15966-15973 and Castells et al., 1994, J. Biol. Chem. 269:8393-8401). One of these (gp49B1) predicts a 35-kDa protein core that contains two C2-type, immunoglobulin (Ig)-like domains in the

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[1] The abbreviations used are: BSA, bovine serum albumin; FcαR, Fc receptor for IgA; FcεRI, high affinity Fc receptor for IgE; Fcγ2R, Fc receptor for IgG2; FcγRIIb1 and FcγRIIb2, Fc receptors for IgG, type IIb1 and IIb2, respectively; FITC, fluorescein isothiocyanate; H/B/A, HBSS containing 0.1% (w/v) BSA and 0.02% (w/v) sodium azide; HBSS, calcium- and magnesium-free Hanks' Balanced Salt Solution; Ig, immunoglobulin; ITIM, immunoreceptor tyrosine-based inhibition motif; KIR, killer cell inhibitory receptor; LT, leukotriene; mAb, monoclonal antibody; mBMMC, mouse bone marrow-derived mast cells; mMIR, mouse mast cell inhibitory receptor; MHC, major histocompatibility complex; NK, natural killer; PBS, phosphate buffered saline; PTP, protein tyrosine phosphatase; RT, reverse transcriptase; TPBS, 1% Tween 20 in PBS; WCM, WEHI-3 cell conditioned medium.

extracellular region (Castells et al., 1994, J. Biol. Chem. 269:8393-8401). The expression of the gp49B1 cDNA in COS transfectants confers binding of mAb B23.1, indicating that the mAb recognizes an epitope present on gp49B1 (Castells et al., 1994, J. Biol. Chem. 269:8393-8401). One of the other cDNAs (gp49A) isolated in the same screen predicts a 32-kDa protein core that has two C2-type, Ig-like domains in its extracellular region and is 89% identical at the amino acid level to gp49B1. gp49A also has 100% amino acid identity with gp49B1 in the transmembrane and 88% identity in the first 34 amino acids of the cytoplasmic domain. The cytoplasmic region of gp49A then diverges for 8 amino acids because of a frameshift in the nucleotide sequence and terminates so that it is 32 amino acids shorter than gp49B1. Each of the above-referenced articles is hereby incorporated by reference.

Summary of the Invention

We have discovered a family of human gp49 molecules. We have also discovered that mammalian gp49 molecules (particularly human gp49 molecules) are directly involved in downregulating FcεRI-mediated release of proinflammatory mediators from mast cells. In addition to the specific human and mouse gp49 sequences provided (or cross-referenced) herein, relevant molecules include fragments of those sequences and related polypeptides described elsewhere herein. These polypeptides (and non-peptide mimetics), as well as nucleic acid encoding them, can be used for a variety of therapies, particularly therapies involving control of mast cell activation and control of inflammatory and allergic responses. Mammalian (particularly human) gp49 polypeptides can be used to verify, evaluate or mimic

immunological processes, as well as the effect of exogenous substances on such processes.

One embodiment of the invention generally features a substantially pure human gp49 polypeptide, particularly polypeptides that are soluble in physiological fluid. Also featured are polypeptides comprising at least one of the following functional human gp49 domains: a) a human gp49 signal peptide domain; b) a human gp49 C-2 Ig domain; c) a human gp49 transmembrane domain; d) a human gp49 cytoplasmic tail (e.g., polypeptides comprising an ITIM motif of a human gp49 cytoplasmic tail. Preferably, the gp49 polypeptide includes an amino acid sequence substantially identical to the amino acid sequence shown in Fig. 1 or the amino acid sequence shown in Fig. 2.

By "isolated nucleic acid" is meant DNA that is not immediately contiguous with both of the coding sequences with which it is immediately contiguous (one on the 5′ end and one on the 3′ end) in the naturally occurring genome of the organism from which it is derived. Thus, a recombinant nucleic acid could include some or all of the 5′ non-coding (e.,g., promoter) sequences which are immediately contiguous to the coding sequence. The term therefore includes, for example, a recombinant DNA which is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequence.

By "mature human gp49" is meant a polypeptide having the sequence shown in FIG's 1 or 2 lacking the leader sequence. Polypeptides substantially identical to

mature human gp49 have an amino acid sequence which is at least 85%, preferably 90%, and most preferably 95% or even 99% identical to the amino acid sequence of the gp49 polypeptide of the FIGs.

By "substantially identical" is meant a polypeptide or nucleic acid having a sequence that is at least 85%, preferably 90%, and more preferably 95% or more identical to the sequence of the reference amino acid or nucleic acid sequence (e.g. of HM18 or HM43). For polypeptides, the length of the reference polypeptide sequence will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably 35 amino acids. For nucleic acids, the length of the reference nucleic acid sequence will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

Sequence identity can be measured using sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, WI 53705).

In the case of polypeptide sequences which are less than 100% identical to a reference sequence, the non-identical positions are preferably, but not necessarily, conservative substitutions for the reference sequence. Conservative substitutions typically include substitutions within the following groups: glycine and alanine; valine, isoleucine, and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenylalanine and tyrosine.

Where a particular polypeptide is said to have a specific percent identity to a reference polypeptide of a

defined length, the percent identity is relative to the reference peptide. Thus, a peptide that is 50% identical to a reference polypeptide that is 100 amino acids long can be a 50 amino acid polypeptide that is completely identical to a 50 amino acid long portion of the reference polypeptide. It might also be a 100 amino acid long polypeptide which is 50% identical to the reference polypeptide over its entire length. Of course, many other polypeptides will meet the same criteria.

Polypeptides corresponding to one or more domains of gp49 are also within the scope of the invention. Preferred polypeptides are those which are soluble under normal physiological conditions. Also within the invention are soluble fusion proteins in which a full-length form of gp49 or a portion (e.g., one or more domains) thereof is fused to an unrelated protein or polypeptide (i.e., a fusion partner) to create a fusion protein.

The invention also features isolated nucleic acid sequences that encode a portion of gp49 and various functional domains of gp49. Also within the invention are nucleic acids encoding polypeptides corresponding to one or more domains of gp49. Preferred nucleic acids encode polypeptides that are soluble under normal physiological conditions. Also within the invention are nucleic acids encoding fusion proteins in which a portion of gp49 or a portion (e.g., one or more domains) thereof is fused to an unrelated protein or polypeptide (i.e., a fusion partner) to create a fusion protein.

Encompassed within the invention are nucleic acid sequences that encode forms of gp49 in which sequences are altered or deleted.

The nucleic acids of the invention include nucleic acids encoding mature gp49 as well as gp49 polypeptides fused to a polypeptide which facilitates secretion, e.g.,

a secretory sequence. Such a fused protein is typically referred to as a preprotein. The secretory sequence can be removed by the host cell to form the mature protein. Also within the invention are nucleic acids that encode mature gp49 fused to a polypeptide sequence to produce an inactive preprotein. Preproteins can be converted into the active form of the protein by removal of the inactivating sequence.

The invention also encompasses nucleic acids that hybridize under stringent conditions to a nucleic acid encoding a gp49 polypeptide. "Stringent conditions" means hybridization at 50°C in Church buffer (7% SDS, 0.5% $NaHPO_4$, 1mM EDTA,1%BSA) and washing at 50°C in 2x SSC. The hybridizing portion of the hybridizing nucleic acids are preferably 20, 30, 50, or 70 bases long. Preferably, the hybridizing portion of the hybridizing nucleic acid is 95% or even 98% identical to the sequence of a portion of a nucleic acid encoding a gp49 polypeptide. Hybridizing nucleic acids of the type described above can be used as a cloning probe, a primer (e.g., a PCR primer), or a diagnostic probe. Preferred hybridizing nucleic acids encode a polypeptide having some or all of the biological activities possessed by naturally-occurring gp49. Hybridizing nucleic acids can be splice variants encoded by one of the gp49 genes described herein. Thus, they may encode a protein which is shorter or longer than the various forms of gp49 described herein. Hybridizing nucleic acids may also encode proteins which are related to gp49 (e.g, proteins encoded by genes which include a portion having a relatively high degree of identity to a gp49 gene described herein).

The invention also features substantially pure gp49 polypeptides. Among the polypeptides encompassed within the invention are those corresponding to the

extracellular domain, the transmembrane domain, the cytoplasmic domain, and various functional domains of gp49.

The invention also encompasses polypeptides and nucleic acids whose sequences are substantially identical to that of a form of gp49 described herein.

By "protein" and "polypeptide" is meant any chain of amino acids, regardless of length or post-translational modification (e.g., glycosylation or phosphorylation).

By "substantially pure" is meant a preparation which is at least 60% by weight (dry weight) the compound of interest, i.e., a gp49 polypeptide. Preferably the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight the compound of interest. Purity can be measured by any appropriate standard method, e.g., column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

The term "nucleic acid" encompasses both RNA and DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid may be double-stranded or single-stranded. Where single-stranded, the nucleic acid may be the sense strand or the antisense strand.

The polypeptides of the invention include, but are not limited to: recombinant polypeptides, natural polypeptides, and synthetic polypeptides as well as polypeptides which are preproteins or proproteins.

The polypeptides of the invention can be expressed fused to another polypeptide, e.g., a marker polypeptide or fusion partner. For example, the polypeptide can be fused to a hexa-histidine tag to facilitate purification of bacterially expressed protein or a hemagglutinin tag to facilitate purification of protein expressed in eukaryotic cells.

The invention features transformed cells harboring a nucleic acid encompassed by the invention. The invention also features vectors and plasmid which include a nucleic acid of the invention which is properly positioned for expression.

By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced, by means of recombinant DNA techniques, a DNA molecule encoding (as used herein) gp49 polypeptide.

By "positioned for expression" is meant that the selected DNA molecule is positioned adjacent to one or more sequence elements which direct transcription and/or translation of the sequence such that the sequence elements can control transcription and/or translation of the selected DNA (i.e., the selected DNA is operably associated with the sequence elements). Such operably associated elements can be used to facilitate the production of a gp49 polypeptide.

The invention also features purified antibodies which specifically bind a gp49 protein or polypeptide.

By "purified antibody" is meant an antibody which is at least 60%, by dry weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the preparation is at least 75%, more preferably at least 90%, and most preferably at least 99%, by dry weight, antibody.

By "specifically binds" is meant an antibody that recognizes and binds to a particular antigen, e.g., gp49 polypeptide, but which does not substantially recognize and bind to other molecules in a sample, e.g., a biological sample, which naturally includes gp49.

The invention also features antagonists and agonists of gp49. Antagonists can inhibit one or more of the functions of gp49. Suitable antagonists can include large or small molecules, antibodies to gp49, and gp49

polypeptides which compete with a native form of gp49. Agonists of gp49 will enhance or facilitate one or more of the functions of gp49. Suitable agonists can include, for example, large or small molecules and antibodies to gp49.

The invention also features a substantially pure polypeptide which includes a first portion and a second portion; the first portion includes a gp49 polypeptide and the second portion includes, e.g.,

The invention also features a substantially pure polypeptide which includes a first portion and a second portion; the first portion includes a gp49 polypeptide the said second portion includes a detectable marker.

In another aspect the invention features a recombinant nucleic acid encoding a gp49 polypeptide. In various preferred embodiments the nucleic acid encodes a soluble gp49 polypeptide.

The invention also features a nucleic acid encoding a hybrid polypeptide. This hybrid polypeptide includes a first portion and a second portion; the first portion includes a gp49 polypeptide; the second portion includes a FcϵRI region.

The invention also feature a cell which harbors a recombinant nucleic acid encoding a gp49 polypeptide; a vector which includes a nucleic acid encoding a gp49 polypeptide.

In another aspect the invention features an antibody which selectively binds to a gp49 polypeptide. In a preferred embodiment the antibody is a monoclonal antibody.

The invention also features a pharmaceutical composition which includes a gp49 polypeptide.

The invention features a method for detecting inflammation. This method includes: (a) obtaining a biological sample; (b) contacting the sample with an

antibody which selectively binds a gp49 polypeptide; and (c) determining the amount of the antibody selectively bound to said biological sample as a measure of inflammation.

In another aspect, the invention features a method for treating inflammation in a patient which includes administering to the patient an agonist of gp49. Specific medical indications include allergic rhinitis, subacute and chronic urticaria, and bronchial asthma.

The invention also features administering to the patient a substantially pure gp49 polypeptide capable of inhibiting mast cell activity.

Also featured are recombinant nucleic acids encoding a human gp49 polypeptides and fusion polypeptides, as well as cells or vectors comprising the recombinant nucleic acids.

The invention also features pharmaceutical compositions comprising the gp49 polypeptides or the nucleic acids.

The invention also features methods of treating an undesired immune response (particularly mast cell related diseases) in a patient by administering a mammalian gp49 polypeptide or nucleic acid encoding a mammalian gp49 polypeptide.

Other features and advantages of the invention will be apparent from the following detailed descriptions, and from the claims.

Brief Description of the Drawings

Fig. 1 is a depiction of the nucleotide and amino acid sequences of a form of human gp49 (HM18), including a putative signal sequence.

Fig. 2 is a depiction of the nucleotide and amino acid sequences of a form of human gp49 (HM43), including a putative signal sequence.

Fig. 3 compares the sequence of HM18 to the sequence of mouse gp41B1.

Detailed Description

The invention encompasses, but is not limited to, gp49 proteins and polypeptides that are functionally related to gp49 encoded by the nucleotide sequence of Fig. 1 or Fig. 2. Functionally related proteins and polypeptides include any protein or polypeptide sharing a functional characteristic with gp49, e.g., the ability to affect proliferation, differentiation, survival, apoptosis, or activation of a cell type whose proliferation, differentiation, survival, apoptosis, or activation is affected by gp49. Such functionally related gp49 polypeptides include, but are not limited to, additions or substitutions of amino acid residues within the amino acid sequence encoded by the gp49 sequences described herein which result in a silent change, thus producing a functionally equivalent gene product. Amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues involved.

For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan, and methionine; polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine; positively charged (basic) amino acids include arginine, lysine, and histidine; and negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

While random mutations can be made to gp49 DNA (using random mutagenesis techniques well known to those skilled in the art) and the resulting mutant gp49 proteins can be tested for activity, site-directed mutations of the gp49 coding sequence can be engineered

(using site-directed mutagenesis techniques well known to those skilled in the art) to generate mutant gp49s with increased function, e.g., greater stimulation of cell proliferation, or decreased function, e.g., lesser stimulation of cell proliferation.

To design functionally related and functionally variant gp49 polypeptides, it is useful to distinguish between conserved positions and variable positions. Fig. 4 shows an alignment between the amino acid sequence of human gp49 and murine gp49, which can be used to determine the conserved and variable amino acid positions.

To preserve gp49 function, it is preferable that conserved residues are not altered. Moreover, alteration of non-conserved residues are preferably conservative alterations, e.g., a basic amino acid is replaced by a different basic amino acid. To produce altered function variants, it is preferable to make non-conservative changes at variable and/or conserved positions. Deletions at conserved and variable positions can also be used to create altered function variants.

Other mutations to the gp49 coding sequence can be made to generate gp49s that are better suited for expression, scale up, etc. in a selected host cell. For example, N-linked glycosylation sites can be altered or eliminated to achieve, for example, expression of a homogeneous product that is more easily recovered and purified from yeast hosts which are known to hyperglycosylate N-linked sites. To this end, a variety of amino acid substitutions at one or both of the first or third amino acid positions of any one or more of the glycosylation recognition sequences which occur (in N-X-S or N-X-T), and/or an amino acid deletion at the second position of any one or more of such recognition sequences, will prevent glycosylation at the modified

tripeptide sequence. (See , e.g., Miyajima et al., EMBO J. 5:1193, 1986).

Preferred gp49 polypeptides are those polypeptides, or variants thereof, which control mast cell activity. In determining whether a particular gp49 polypeptide or variant thereof controls mast cell activity, one can use any assay techniques disclosed herein or in referenced publications. Preferred gp49 polypeptides and variants have 20%, 40%, 50%, 75%, 80%, or even 90% of the activity of the full-length, mature human form of gp49 described herein. Such comparisons are generally based on equal concentrations of the molecules being compared. The comparison can also be based on the amount of protein or polypeptide required to reach 50% of the maximal stimulation obtainable.

Polypeptides corresponding to one or more domains of gp49. Preferred polypeptides are those which are soluble under normal physiological conditions. Also within the invention are fusion proteins in which a portion (e.g., one or more domains) of gp49 is fused to an unrelated protein or polypeptide (i.e., a fusion partner) to create a fusion protein. The fusion partner can be a moiety selected to facilitate purification, detection, or solubilization, or to provide some other function. Fusion proteins are generally produced by expressing a hybrid gene in which a nucleotide sequence encoding all or a portion of gp49 is joined in-frame to a nucleotide sequence encoding the fusion partner. Also within the scope of the invention are various soluble forms of gp49. For example, the entire extracellular domain of gp49 or a portion thereof can be expressed on its own or fused to a solubilization partner, e.g., an immunoglobulin.

In general, gp49 proteins according to the invention can be produced by transformation

(transfection, transduction, or infection) of a host cell with all or part of a gp49-encoding DNA fragment (e.g., the cDNA described herein) in a suitable expression vehicle. Suitable expression vehicles include: plasmids, viral particles, and phage. For insect cells, baculovirus expression vectors are suitable. The entire expression vehicle, or a part thereof, can be integrated into the host cell genome. In some circumstances, it is desirable to employ an inducible expression vector, e.g., the LACSWITCH™ Inducible Expression System (Stratagene; LaJolla, CA).

Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems can be used to provide the recombinant protein. The precise host cell used is not critical to the invention. The gp49 protein can be produced in a prokaryotic host (e.g., *E. coli* or *B. subtilis*) or in a eukaryotic host (e.g., *Saccharomyces* or *Pichia*; mammalian cells, e.g., COS, NIH 3T3 CHO, BHK, 293, or HeLa cells; or insect cells).

Proteins and polypeptides can also be produced by plant cells. For plant cells viral expression vectors (e.g., cauliflower mosaic virus and tobacco mosaic virus) and plasmid expression vectors (e.g., Ti plasmid) are suitable. Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Rockland, MD; also, <u>see</u>, e.g., Ausubel et al., *Current Protocols in Molecular Biology,* John Wiley & Sons, New York, 1994). The methods of transformation or transfection and the choice of expression vehicle will depend on the host system selected. Transformation and transfection methods are described, e.g., in Ausubel et al. (*Current Protocols in Molecular Biology,* John Wiley & Sons, New York, 1994); expression vehicles may be chosen from those provided, e.g., in *Cloning Vectors*: A

Laboratory Manual (P.H. Pouwels et al., 1985, Supp. 1987).

The host cells harboring the expression vehicle can be cultured in conventional nutrient media adapted as need for activation of a chosen gene, repression of a chosen gene, selection of transformants, or amplification of a chosen gene.

One preferred expression system is the mouse 3T3 fibroblast host cell transfected with a pMAMneo expression vector (Clontech, Palo Alto, CA). pMAMneo provides an RSV-LTR enhancer linked to a dexamethasone-inducible MMTV-LTR promotor, an SV40 origin of replication which allows replication in mammalian systems, a selectable neomycin gene, and SV40 splicing and polyadenylation sites. DNA encoding a gp49 protein would be inserted into the pMAMneo vector in an orientation designed to allow expression. The recombinant gp49 protein would be isolated as described below. Other preferable host cells that can be used in conjunction with the pMAMneo expression vehicle include COS cells and CHO cells (ATCC Accession Nos. CRL 1650 and CCL 61, respectively).

Gp49 polypeptides can be produced as fusion proteins. For example, the expression vector pUR278 (Ruther et al., *EMBO J.* 2:1791, 1983), can be used to create lacZ fusion proteins. The pGEX vectors can be used to express foreign polypeptides as fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can be easily purified from lysed cells by adsorption to glutathione-agarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect cell expression system, Autographa californica nuclear polyhidrosis virus (AcNPV), which grows in Spodoptera frugiperda cells, is used as a vector to express foreign genes. A gp49 coding sequence can be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter, e.g., the polyhedrin promoter. Successful insertion of a gene encoding a gp49 polypeptide or protein will result in inactivation of the polyhedrin gene and production of non-occluded recombinant virus (i.e., virus lacking the proteinaceous coat encoded by the polyhedrin gene). These recombinant viruses are then used to infect spodoptera frugiperda cells in which the inserted gene is expressed (see, e.g., Smith et al., *J. Virol.* 46:584, 1983; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based expression systems can be utilized. In cases where an adenovirus is used as an expression vector, the gp49 nucleic acid sequence can be ligated to an adenovirus transcription/translation control complex, e.g., the late promoter and tripartite leader sequence. This chimeric gene can then be inserted into the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion into a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing a gp49 gene product in infected hosts (see, e.g., Logan, Proc. Natl. Acad. Sci. USA 81:3655, 1984).

Specific initiation signals may also be required for efficient translation of inserted nucleic acid sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where an entire native gp49 gene or CDNA, including its own initiation codon and adjacent sequences, is inserted into the

appropriate expression vector, no additional translational control signals may be needed. In other cases, exogenous translational control signals, including, perhaps, the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators (Bittner et al., Methods in Enzymol. 153:516, 1987).

In addition, a host cell may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in a specific, desired fashion. Such modifications (e.g., glycosylation) and processing (e.g., cleavage) of protein products may be important for the function of the protein. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells that possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product can be used. Such mammalian host cells include, but are not limited to, CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, WI38, and in particular, choroid plexus cell lines.

Alternatively, a gp49 protein can be produced by a stably-transfected mammalian cell line. A number of vectors suitable for stable transfection of mammalian cells are available to the public, see, e.g., Pouwels et

al. (supra); methods for constructing such cell lines are also publicly available, e.g., in Ausubel et al. (supra). In one example, CDNA encoding the gp49 protein is cloned into an expression vector that includes the dihydrofolate reductase (DHFR) gene. Integration of the plasmid and, therefore, the gp49 protein-encoding gene into the host cell chromosome is selected for by including 0.01-300 μM methotrexate in the cell culture medium (as described in Ausubel et al., supra). This dominant selection can be accomplished in most cell types.

Recombinant protein expression can be increased by DHFR-mediated amplification of the transfected gene. Methods for selecting cell lines bearing gene amplifications are described in Ausubel et al. (supra); such methods generally involve extended culture in medium containing gradually increasing levels of methotrexate. DHFR-containing expression vectors commonly used for this purpose include pCVSEII-DHFR and pAdD26SV(A) (described in Ausubel et al., supra). Any of the host cells described above or, preferably, a DHFR-deficient CHO cell line (e.g., CHO DHFR$^-$cells, ATCC Accession No. CRL 9096) are among the host cells preferred for DHFR selection of a stably-transfected cell line or DHFR-mediated gene amplification.

A number of other selection systems can be used, including but not limited to the herpes simplex virus thymidine kinase, hypoxanthine-guanine phosphoribosyl-transferase, and adenine phosphoribosyltransferase genes can be employed in tk, hgprt, or aprt cells, respectively. In addition, gpt, which confers resistance to mycophenolic acid (Mulligan et al., Proc. Natl. Acad. Sci. USA 78:2072, 1981); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin et al., J. Mol. Biol. 150:1, 1981); and hygro, which confers

resistance to hygromycin (Santerre et al., Gene 30:147, 1981), can be used.

Alternatively, any fusion protein can be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described in Janknecht et al., Proc. Natl. Acad. Sci. USA, 88:8972 (1981), allows for the ready purification of non-denatured fusion proteins expressed in human cell lines. In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns, and histidine-tagged proteins are selectively eluted with imidazole-containing buffers.

Alternatively, gp49 or a portion thereof, can be fused to an immunoglobulin Fc domain. Such a fusion protein can be readily purified using an affinity column.

Gp49 proteins and polypeptides can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees, can be used to generate gp49-expressing transgenic animals.

Any technique known in the art can be used to introduce a gp49 transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148, 1985); gene targeting into embryonic stem cells (Thompson et al., Cell 56:313, 1989); and electroporation of embryos (Lo, Mol. Cell. Biol. 3:1803, 1983).

The present invention provides for transgenic animals that carry the gp49 transgene in all their cells, as well as animals that carry the transgene in some, but not all of their cells, i.e., mosaic animals. The transgene can be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems The transgene can also be selectively introduced into and activated in a particular cell type (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232, 1992). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

When it is desired that the gp49 transgene be integrated into the chromosomal site of the endogenous gp49 gene, gene targeting is preferred. Briefly, when such a technique is to be used, vectors containing some nucleotide sequences homologous to an endogenous gp49 gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene also can be selectively introduced into a particular cell type, thus inactivating the endogenous gp49 gene in only that cell type (Gu et al., Science 265:103, 1984). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant gp49 gene can be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA

expression of the transgene in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of gp49 gene-expressing tissue, also can be evaluated immunocytochemically using antibodies specific for the gp49 transgene product.

Once the recombinant gp49 protein is expressed, it is isolated. Secreted forms can be isolated from the culture media, while non-secreted forms must be isolated from the host cells. Proteins can be isolated by affinity chromatography. In one example, an anti-gp49 protein antibody (e.g., produced as described herein) is attached to a column and used to isolate the gp49 protein. Lysis and fractionation of gp49 protein-harboring cells prior to affinity chromatography can be performed by standard methods (see, e.g., Ausubel et al., supra). Alternatively, a gp49 fusion protein, for example, a gp49-maltose binding protein, a gp49-β-galactosidase, or a gp49-trpE fusion protein, can be constructed and used for gp49 protein isolation (see, e.g., Ausubel et al., supra; New England Biolabs, Beverly, MA).

Once isolated, the recombinant protein can, if desired, be further purified, e.g., by high performance liquid chromatography using standard techniques (see, e.g., Fisher, *Laboratory Techniques In Biochemistry And Molecular Biology*, eds., Work and Burdon, Elsevier, 1980).

Polypeptides of the invention, particularly short gp49 fragments, can also be produced by chemical synthesis (e.g., by the methods described in *Solid Phase Peptide Synthesis*, 2nd ed., 1984 The Pierce Chemical Co., Rockford, IL).

These general techniques of polypeptide expression and purification can also be used to produce and isolate useful gp49 fragments or analogs (described herein).

The invention also features proteins which interact with gp49 and are involved in the function of gp49. Also included in the invention are the genes encoding these interacting proteins. Interacting proteins can be identified using methods known to those skilled in the art. One method suitable method is the "two-hybrid system," detects protein interactions *in vivo* (Chien et al., *Proc. Natl. Acad. Sci. USA*, 88:9578, 1991). A kit for practicing this method is available from Clontech (Palo Alto, CA).

Anti-Gp49 Antibodies

Human gp49 proteins and polypeptides (or immunogenic fragments or analogs) can be used to raise antibodies useful in the invention; such polypeptides can be produced by recombinant or peptide synthetic techniques (see, e.g., *Solid Phase Peptide Synthesis*, supra; Ausubel et al., supra). In general, the peptides can be coupled to a carrier protein, such as KLH, as described in Ausubel et al., supra, mixed with an adjuvant, and injected into a host mammal. Antibodies can be purified by peptide antigen affinity chromatography.

In particular, various host animals can be immunized by injection with a gp49 protein or polypeptide. Host animals include rabbits, mice, guinea pigs, and rats. Various adjuvants can be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and

potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*. Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of the immunized animals.

Antibodies within the invention include monoclonal antibodies, polyclonal antibodies, humanized or chimeric antibodies, single chain antibodies, Fab fragments, $F(ab')_2$ fragments, and molecules produced using a Fab expression library.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, can be prepared using the gp49 proteins described above and standard hybridoma technology (see, e.g., Kohler et al., Nature 256:495, 1975; Kohler et al., Eur. J. Immunol. 6:511, 1976; Kohler et al., Eur. J. Immunol. 6:292, 1976; Hammerling et al., In Monoclonal Antibodies and T Cell Hybridomas, Elsevier, NY, 1981; Ausubel et al., supra).

In particular, monoclonal antibodies can be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture such as described in Kohler et al., Nature 256:495, 1975, and U.S. Patent No. 4,376,110; the human B-cell hybridoma technique (Kosbor et al., Immunology Today 4:72, 1983; Cole et al., Proc. Natl. Acad. Sci. USA 80:2026, 1983), and the EBV-hybridoma technique (Cole et al., Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc., pp. 77-96, 1983). Such antibodies can be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the Mab of this invention may be cultivated *in vitro* or *in vivo*. The ability to produce high titers of mAbs *in vivo* makes this the presently preferred method of production.

Once produced, polyclonal or monoclonal antibodies are tested for specific gp49 recognition by Western blot

or immunoprecipitation analysis by standard methods, e.g., as described in Ausubel et al., supra. Antibodies that specifically recognize and bind to gp49 are useful in the invention. For example, such antibodies can be used in an immunoassay to monitor the level of gp49 produced by a mammal (for example, to determine the amount or subcellular location of gp49).

Preferably, antibodies of the invention are produced using fragments of the gp49 protein which lie outside highly conserved regions and appear likely to be antigenic, by criteria such as high frequency of charged residues. In one specific example, such fragments are generated by standard techniques of PCR, and are then cloned into the PGEX expression vector (Ausubel et al., supra). Fusion proteins are expressed in *E. coli* and purified using a glutathione agarose affinity matrix as described in Ausubel, et al., supra.

In some cases it may be desirable to minimize the potential problems of low affinity or specificity of antisera. In such circumstances, two or three fusions can be generated for each protein, and each fusion can be injected into at least two rabbits. Antisera can be raised by injections in a series, preferably including at least three booster injections.

Antisera is also checked for its ability to immunoprecipitate recombinant gp49 proteins or control proteins, such as glucocorticoid receptor, CAT, or luciferase.

The antibodies can be used, for example, in the detection of the gp49 in a biological sample as part of a diagnostic assay. Antibodies also can be used in a screening assay to measure the effect of a candidate compound on expression or localization of gp49. Additionally, such antibodies can be used in conjunction with the gene therapy techniques described to, for

example, evaluate the normal and/or engineered gp49-expressing cells prior to their introduction into the patient. Such antibodies additionally can be used in a method for inhibiting abnormal gp49 activity.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., Proc. Natl. Acad. Sci., 81:6851, 1984; Neuberger et al., Nature, 312:604, 1984; Takeda et al., Nature, 314:452, 1984) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine Mab and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Patent 4,946,778; and U.S. Patents 4,946,778 and 4,704,692) can be adapted to produce single chain antibodies against a gp49 protein or polypeptide. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Antibody fragments that recognize and bind to specific epitopes can be generated by known techniques. For example, such fragments include but are not limited to $F(ab')_2$ fragments that can be produced by pepsin digestion of the antibody molecule, and Fab fragments that can be generated by reducing the disulfide bridges of $F(ab')_2$ fragments. Alternatively, Fab expression libraries can be constructed (Huse et al., Science, 246:1275, 1989) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

Antibodies to the gp49 can, in turn, be used to generate anti-idiotype antibodies that resemble a portion of gp49 using techniques well known to those skilled in the art (see, e.g., Greenspan et al., FASEB J. 7:437, 1993; Nissinoff, J. Immunol. 147:2429, 1991). For example, antibodies that bind to gp49 and competitively inhibit the binding of a ligand of gp49 can be used to generate anti-idiotypes that resemble a ligand binding domain of gp49 and, therefore, bind and neutralize a ligand of gp49. Such neutralizing anti-idiotypic antibodies or Fab fragments of such anti-idiotypic antibodies can be used in therapeutic regimens.

Oligonucleotides therapeutic agents can be DNA or RNA or chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. The oligonucleotide can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, hybridization, etc. The oligonucleotide may include other appended groups such as peptides (e.g., for targeting host cell receptors *in vivo*), or agents facilitating transport across the cell membrane (as described, e.g., in Letsinger et al., Proc. Natl. Acad. Sci. USA 86:6553, 1989; Lemaitre et al., Proc. Natl. Acad. Sci. USA 84:648, 1987; PCT Publication No. WO 88/09810) or the blood-brain barrier (see, e.g., PCT Publication No. WO 89/10134), or hybridization-triggered cleavage agents (see, e.g., Krol et al., BioTechniques 6:958, 1988), or intercalating agents (see, e.g., Zon, Pharm. Res. 5:539, 1988). To this end, the oligonucleotide can be conjugated to another molecule, e.g., a peptide, hybridization triggered cross-linking agent, transport agent, or hybridization-triggered cleavage agent.

The oligonucleotide may comprise at least one modified base moiety which is selected from the group

including, but not limited to, 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthine, xantine, 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethyl-aminomethyluracil, dihydrouracil, beta-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-theouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), 5-methyl-2-thiouracil, 2-(3-amino-3-N-2-carboxypropl) uracil, (acp3)w, and 2,6-diaminopurine.

The oligonucleotide may also comprise at least one modified sugar moiety selected from the group including, but not limited to, arabinose, 2-fluoroarabinose, xylulose, and hexose.

In yet another embodiment, the oligonucleotide comprises at least one modified phosphate backbone selected from the group consisting of a phosphorothioate, a phosphorodithioate, a phosphoramidothioate, a phosphoramidate, a phosphordiamidate, a methylphosphonate, an alkyl phosphotriester, and a formacetal, or an analog of any of these backbones.

In yet another embodiment, the oligonucleotide is an α-anomeric oligonucleotide. An α-anomeric oligonucleotide forms specific double-stranded hybrids with complementary RNA in which, contrary to the usual β-units, the strands run parallel to each other (Gautier et

al., Nucl. Acids. Res. 15:6625, 1987). The oligonucleotide is a 2'-0-methylribonucleotide (Inoue et al., Nucl. Acids Res. 15:6131, 1987), or a chimeric RNA-DNA analog (Inoue et al., FEBS Lett. 215:327, 1987).

Oligonucleotides of the invention can be synthesized by standard methods known in the art, e.g. by use of an automated DNA synthesizer (such as are commercially available from Biosearch, Applied Biosystems, etc.). As examples, phosphorothioate oligonucleotides can be synthesized by the method of Stein et al. (Nucl. Acids Res. 16:3209, 1988), and methylphosphonate oligonucleotides can be prepared by use of controlled pore glass polymer supports (Sarin et al., Proc. Natl. Acad. Sci. USA 85:7448, 1988).

The nucleic acid molecules should be delivered to cells that express gp49 *in vivo*, e.g., brain, heart, kidney, lung, uterus, endothelial cells, fibroblasts, and bone marrow stromal cells. A number of methods have been developed for delivering DNA or RNA to cells; e.g., molecules can be injected directly into the tissue site, or modified molecules, designed to target the desired cells (e.g., linked to peptides or antibodies that specifically bind receptors or antigens expressed on the target cell surface) can be administered systemically.

If intracellular concentrations of the molecule sufficient to suppress translation of endogenous mRNAs are not immediately achieved, a preferred approach uses a recombinant DNA construct in which the oligonucleotide is placed under the control of a strong *pol* III or *pol* II promoter. The use of such a construct to transfect target cells in the patient will result in the transcription of sufficient amounts of single stranded RNAs that will form complementary base pairs with the endogenous gp49 transcripts and thereby prevent translation of the gp49 MRNA. For example, a vector can

be introduced *in vivo* such that it is taken up by a cell and directs the transcription of an RNA. Such a vector can remain episomal or become chromosomally integrated, as long as it can be transcribed to produce the desired RNA.

Such vectors can be constructed by recombinant DNA technology methods standard in the art. Vectors can be plasmid, viral, or others known in the art, used for replication and expression in mammalian cells. Expression of the sequence encoding the RNA can be by any promoter known in the art to act in mammalian, preferably human cells. Such promoters can be inducible or constitutive. Such promoters include, but are not limited to: the SV40 early promoter region (Bernoist et al., Nature 290:304, 1981); the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto et al., Cell 22:787-797, 1988); the herpes thymidine kinase promoter (Wagner et al., Proc. Natl. Acad. Sci. USA 78:1441, 1981); or the regulatory sequences of the metallothionein gene (Brinster et al., Nature 296:39, 1988).

Any type of plasmid, cosmid, YAC, or viral vector can be used to prepare the recombinant DNA construct which can be introduced directly into the tissue site; e.g., the brain, heart, kidney, lung, uterus, endothelial cells, fibroblasts, and bone marrow stromal cells. Alternatively, viral vectors can be used that selectively infect the desired tissue (e.g., for brain, herpesvirus vectors may be used), in which case administration can be accomplished by another route (e.g., systemically).

Alternatively, endogenous gp49 gene expression can be reduced by targeting deoxyribonucleotide sequences complementary to the regulatory region of the gp49 gene (i.e., the gp49 promoter and/or enhancers) to form triple helical structures that prevent transcription of the gp49

gene in target cells in the body (Helene Anticancer Drug Des. 6:569, 1981; Helene et al., Ann. N.Y. Accad. Sci. 660:27, 1992; and Maher, Bioassays 14:807, 1992).

The Identification of Proteins which Interact with Gp49

The invention also features proteins which interact with gp49. Any method suitable for detecting protein-protein interactions may be employed for identifying transmembrane proteins, intracellular, or extracellular proteins that interact with gp49. Among the traditional methods which may be employed are co-immunoprecipitation, crosslinking and co-purification through gradients or chromatographic columns of cell lysates or proteins obtained from cell lysates and the use of gp49 to identify proteins in the lysate that interact with the gp49. For these assays, the gp49 polypetide can be a full length gp49, a soluble extracellular domain of gp49, or some other suitable gp49 polypeptide. Once isolated, such an interacting protein can be identified and cloned and then used, in conjunction with standard techniques, to identify proteins with which it interacts. For example, at least a portion of the amino acid sequence of a protein which interacts with the gp49 can be ascertained using techniques well known to those of skill in the art, such as via the Edman degradation technique. The amino acid sequence obtained may be used as a guide for the generation of oligonucleotide mixtures that can be used to screen for gene sequences encoding the interacting protein. Screening may be accomplished, for example, by standard hybridization or PCR techniques. Techniques for the generation of oligonucleotide mixtures and the screening are well-known. (Ausubel, *supra;* and PCR Protocols: A Guide to Methods and Applications, 1990, Innis et al., eds. Academic Press, Inc., New York).

Additionally, methods may be employed which result directly in the identification of genes which encode proteins which interact with gp49. These methods include, for example, screening expression libraries, in a manner similar to the well known technique of antibody probing of λgt11 libraries, using labeled gp49 poypeptide or a gp49 fusion protein, e.g., an gp49 polypeptide or domain fused to a marker such as an enzyme, fluorescent dye, a luminescent protein, or to an IgFc domain.

There are also methods which are capable of detecting protein interaction. A method which detects protein interactions *in vivo* is the two-hybrid system (Chien et al., *Proc. Natl. Acad. Sci. USA*, 88:9578, 1991). A kit for practicing this method is available from Clontech (Palo Alto, CA).

Briefly, utilizing such a system, plasmids are constructed that encode two hybrid proteins: one plasmid includes a nucleotide sequence encoding the DNA-binding domain of a transcription activator protein fused to a nucleotide sequence encoding gp49, a gp49 polypeptide, or a gp49 fusion protein, and the other plasmid includes a nucleotide sequence encoding the transcription activator protein's activation domain fused to a CDNA encoding an unknown protein which has been recombined into this plasmid as part of a CDNA library. The DNA-binding domain fusion plasmid and the CDNA library are transformed into a strain of the yeast *Saccharomyces cerevisiae* that contains a reporter gene (e.g., HBS or *lacZ*) whose regulatory region contains the transcription activator's binding site. Either hybrid protein alone cannot activate transcription of the reporter gene: the DNA-binding domain hybrid cannot because it does not provide activation function and the activation domain hybrid cannot because it cannot localize to the activator's binding sites. Interaction of the two hybrid

proteins reconstitutes the functional activator protein and results in expression of the reporter gene, which is detected by an assay for the reporter gene product.

The two-hybrid system or related methodology may be used to screen activation domain libraries for proteins that interact with the "bait" gene product. By way of example, and not by way of limitation, gp49 may be used as the bait gene product. Total genomic or CDNA sequences are fused to the DNA encoding an activation domain. This library and a plasmid encoding a hybrid of bait gp49 gene product fused to the DNA-binding domain are cotransformed into a yeast reporter strain, and the resulting transformants are screened for those that express the reporter gene. For example, a bait gp49 gene sequence, such as gp49 or a domain of gp49 can be cloned into a vector such that it is translationally fused to the DNA encoding the DNA-binding domain of the GAL4 protein. These colonies are purified and the library plasmids responsible for reporter gene expression are isolated. DNA sequencing is then used to identify the proteins encoded by the library plasmids.

A CDNA library of the cell line from which proteins that interact with bait gp49 gene product are to be detected can be made using methods routinely practiced in the art. According to the particular system described herein, for example, the CDNA fragments can be inserted into a vector such that they are translationally fused to the transcriptional activation domain of GAL4. This library can be co-transformed along with the bait gp49 gene-GAL4 fusion plasmid into a yeast strain which contains a lacZ gene driven by a promoter which contains GAL4 activation sequence. A CDNA encoded protein, fused to GAL4 transcriptional activation domain, that interacts with bait gp49 gene product will reconstitute an active GAL4 protein and thereby drive expression of the HIS3

gene. Colonies which express HIS3 can then be purified from these strains, and used to produce and isolate the bait gp49 gene-interacting protein using techniques routinely practiced in the art.

Gp49 Proteins and Polypeptides

Gp49 proteins and polypeptides and gp49 fusion proteins can be prepared for a wide range of uses including, but not limited to, generation of antibodies, preparation of reagents for diagnostic assays, identification of other molecules involved in inflammation (particularly brain inflammation), preparation of reagents for use in screening assays for inflammatory modulators, and preparation of therapeutic agents for treatment of inflammation-related disorders.

We have cloned human CDNAS homologous to mouse gp49. One of the resulting human gp49 cDNAs is a 1.6kb cDNA designated HM18, which predicts a ~49.3 kd type 1 integral membrane protein with two C-2 type immunoglobulin superfamily domains and two consensus ITIM motifs in the cytoplasmic domain. HM18 shares 62% nucleotide identity and 47% amino acid identity (57% similarity) with mouse gp49. Similarity is greatest in the first C-2 domain, with 72% nucleotide and 62% amino acid identity (69% similarity). The ITIM motifs of gp4(B1 are conserved in HM18. The gene for HM18 is localized to human chromosome 19 (19q13.4). RNA blotting and SDS-PAGE immunoblotting reveal transcripts and protein for HM18 in peripheral blood monocytes.

A second cDNA (HM43) was isolated which encodes a 41.6kd protein with four C-2 domains, but no transmembrane or cytoplasmic domains, suggesting that it encodes a soluble form of gp49.

Several other cDNAs have been isolated and partially characterized, indicating the presence of other gene(s) encoding protein(s) with four C-2 domains, a

transmembrane domain, and a cytoplasmic tail that contains the conserved ITIM motifs.

In sum, in mouse two separate genes have been described for gp49: gp49A nd gp49B, respectively. In man, there is a multigene family, with at least three separate genes, as indicated by the existence of several homologous but distinct cDNAs, data characterizing P1 artificial chromosome (PAC) clones of human genomic DNA, adn genomic southern blotting.

MATERIALS AND METHODS

ALIGN Comparison of the gp49B1 Amino Acid Sequence with Other Members of the Ig Superfamily. Full-length sequences were aligned with the dynamic programming algorithm of Altschul and Ericson (Altschul et al., 1986, Bull. Math. Biol. 48:603-616), and a similarity score was derived with the Dayhoff cost matrix (Dayhoff et al., 1983, Methods Enzymol. 91:524-545), with a cost of 6 for opening a gap (Williams et al., 1988, Annu. Rev. Immunol. 6:381-405) and an incremental cost of 0.5 for each residue in the gap. To determine the significance of the similarity score, the sequence being compared with gp49B1 was then randomized 100 times; and the gp49B1 sequence was aligned with each randomized sequence. The means ± standard deviations of the resulting similarity scores were calculated. To determine the significance of the original comparison, its similarity score was subtracted from the mean score of the randomized comparisons and the result was divided by the standard deviation of the randomized comparisons to obtain the ALIGN score (Altschul et al., 1986, Bull. Math. Biol. 48:603-616). Scores ≥3 indicate that the similarities between the compared sequences are significantly greater than would occur by chance alone (Williams et al., 1988, Annu. Rev. Immunol. 6:381-405).

Generation of mBMMC and Stable gp49 Transfectants in P815 Cells. Bone marrow cells from male BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) were cultured for 3-7 wk in enriched medium containing 50% WEHI-3 cell (American Type Culture Collection, ATCC) conditioned medium (WCM) as described (Razin et al., 1984, J. Immunol. 132:1479-1486). After 3 wk, >97% of the non-adherent cells in the cultures were mast cells as assessed by metachromatic staining with toluidine blue (Razin et al., 1983, J. Exp. Med. 157:189-201).

To prepare transfectants, gp49A and gp49B1 cDNAs were subjected to PCR with primers whose sequences matched nucleotides 22 to 42 (5'-CGAACATTGCCTGGACTCACC-3'; 5' untranslated region) and 1288 to 1309 (5'-GTTTCTCATTAGATGACTTG-3'; 3' untranslated region) that are common to the gp49A and gp49B1 cDNAs (Castells et al., 1994, J. Biol. Che. 269:8393-8401). The 5' ends of the upstream and downstream primers contained *Xba*I and *Bam*HI linkers, respectively, to facilitate ligation into the mammalian expression vector, pMH-NEO (Hahn et al., 1993, Gene 127:267-268). PCR was performed for 30 cycles with primer annealing at 60°C. After ligation of the amplified cDNAs into pMH-NEO, DH5α bacterial cells (Stratagene, La Jolla, CA) were transformed either with pMH-NEO alone or with pMH-NEO containing gp49A cDNA or gp49B1 cDNA. Plasmid DNA containing the vector alone or with the inserted cDNAs was purified with Nucleobond AX (The Nest Group, Southborough, MA) and introduced into P815 cells by electroporation with 200 mV and 960 μfarads in a BioRad Gene Pulser Electroporator. Stable transfectants were selected by their resistance to 1.2 mg/ml G418.

After ≈6 weeks of culture, gp49B1 transfectants were stained with mAb B23.1 and $F(ab')_2$ fragments of fluorescein isothiocyanate (FITC)-labeled goat anti-rat

IgM (Jackson Immunoresearch, Avondale, PA). The cells were sorted, and the 1% brightest cells were collected and cloned by being seeded in 96-well plates at an average density of 0.3 cells/well. After 7 days, the plates in which cells were growing in fewer than 30% of the wells were analyzed by flow cytometry for binding of mAb B23.1. Five clones exhibited similar or stronger staining with mAb B23.1 compared with BMMC, and one such clone (1-11G) was chosen for study.

For identification of gp49A transfectants, total RNA was isolated from stable P815 clones that had been transfected with gp49A cDNA, and the presence of gp49A transcripts was detected by reverse transcriptase (RT)-PCR. For the RT step, a downstream primer located in exon 6 of gp49A was used (5'-TAGCCTTATTTTTGTGACGAC-3'). PCR was performed on the resultant cDNAs with the same downstream primer plus an upstream primer from exon 3 of gp49A (5'-ACCAAGTTCAAAATTCGATTT-3'); an annealing temperature of 60°C was used for 40 cycles. These primers were specific for gp49A because they generated a product by RT-PCR from COS-7 cells transiently transfected with a gp49A, but not gp49B1, cDNA (data not shown). Four stable P815 clones expressed high steady-state levels of gp49A mRNA, and one (clone 9) was selected for study.

Preparation of Antibodies. Monoclonal rat IgE anti-dinitrophenyl hapten (clone LO-DNP-30) was obtained from Zymed (South San Francisco, CA). Rat IgM mAb B23.1 anti-mouse gp49B1 and rat IgM mAb RATNP 17.3 were purified by affinity chromatography from ascites produced in BALB/c nu/nu mice as described (Katz et al., 1989, J. Immunol. 142:919-926). For production of antibody to gp49A, a synthetic peptide (C<u>E</u>VPLE<u>N</u>RNK<u>T</u>KF<u>K</u>I<u>RF</u>; prepared at the Brigham and Women's Hospital Protein Microsequ ncing Laboratory) was synthesized corresponding

to amino acids 72-87 of the extracellular domain of gp49A; this peptide differs from the sequence of gp49B1 at the underlined amino acids (Castells et al., 1994, J. Biol. Chem. 269:8393-8401). The amino-terminal cysteine is not found in the gp49A amino acid sequence but was added to facilitate coupling to carrier protein. The peptide (2 mg) was coupled to 2 mg of maleimide-activated keyhole limpet hemocyanin (Imject Kit; Pierce, Rockford IL). The conjugate (125 μg in 250 μl phosphate-buffered saline [PBS], pH 7.2) was mixed with an equal volume of Hunter's Titermax adjuvant emulsion (CytRx Co., Norcross, GA) and injected intramuscularly at 4 sites in each rabbit; a booster immunization consisting of 100 μg of each peptide conjugate and the same adjuvant was administered 3 weeks later and 2 wk after the booster immunization blood was collected.

The titer of the antiserum was determined by ELISA. Peptides (10 μg in PBS, pH 7.5) were incubated for 1 h at room temperature in 96-well Immulon II plates (Dynatech Labs, Chantilly, VA). The wells were washed 3 times with PBS, blocked by incubation with 1% bovine serum albumin (BSA) in PBS (BSA/PBS) for 1 h at room temperature, washed 3 times with 1% Tween 20 in PBS (TPBS), and incubated with serial dilutions of rabbit serum in 1% BSA/PBS for 1 h at room temperature. The wells were washed 5 times with TPBS, incubated for 1 h at room temperature with a 1:2000 dilution of horseradish peroxidase-labeled goat-anti-rabbit IgG (Biorad, Hercules, CA) in 1% BSA/PBS, washed 6 times with TPBS, and incubated for 20 min at room temperature with 100 μl of a 200 μg/ml solution of 2,2'-Azino-di-[3-ethylbenzythiazolinsulfonate] (Boehringer-Mannheim, Indianapolis, IN) containing 0.015% hydrogen peroxide in citrate buffer, pH 4.25. Absorbance at 405 nm was measured on a Titertek Multiscan MCC/340 plate reader.

Half-maximal binding of anti-gp49A_{72-87} serum to the immunizing peptide occurred at an ~1200-fold dilution of serum. The reactivity of anti-gp49A_{72-87} serum with a peptide consisting of a cysteine plus gp49B1 amino acids 72-87 (CQVPLETRNKAKFNIPS) was ~10% of its reactivity with the immunizing peptide, as defined by reactivity with dilutions of the serum.

Anti-gp49_{72-87} Ig was purified on peptide affinity columns. Each peptide (10 mg) was coupled to 1 mg cyanogen bromide-activated Sepharose 4B (Pharmacia-LKB Biotechnology, Piscataway, NJ), according to the manufacturer's protocol. An 8-ml sample of anti-gp49_{72-87} serum was applied to a column containing 2 ml of resin coupled with the heterologous gp49$B1_{72-87}$ peptide, and the flow-through was applied to a column containing the homologous peptide. The second column was washed sequentially with 20 column volumes of 10 mM Tris-HCl, pH 7.5; 20 column volumes of 500 mM NaCl in 10 mM Tris-HCl, pH 7.5; and 20 column volumes of 10 mM Tris-HCl, pH 7.5. Ig was then eluted with 2 column volumes of 100 mM glycine, pH 2.5 into 0.2-column volumes of 1 M Tris-HCl, pH 8.0 and was dialyzed twice against PBS, pH 7.2. Non-immune Ig was purified on a Protein A-Sepharose column with the same steps described above.

Flow Cytometric Analyses. Samples containing 2 to 2.5 x 10^5 cells were pelleted into tubes, and the cells were resuspended in 25 μl of either 20 μg/ml mAb B23.1 or an equal concentration of rat myeloma IgM in calcium- and magnesium-free Hanks' Balanced Salt Solution (HBSS) containing 0.1% (w/v) BSA and 0.02% (w/v) sodium azide (H/B/A). The cells were then incubated for 30 min at 4°C, washed by centrifugation in cold H/B/A, and incubated for 30 min at 4°C in a saturating concentration of FITC-labeled $F(ab')_2$ fragments of goat anti-rat IgM (μ chain specific). To stain cells with anti-gp49A_{72-87} IgG,

an analogous procedure was used with 90 μg/ml anti-gp49A$_{72-87}$ IgG or an equal concentration of non-immune rabbit Ig in the first step. A saturating concentration of FITC-labeled F(ab')$_2$ fragments of goat anti-rabbit IgG heavy and light chains was used as the second antibody. All second antibodies were obtained from Jackson Immunoresearch. After incubation with second antibodies, the cells were washed by centrifugation, fixed in 2% paraformaldehyde, and analyzed with a Becton Dickinson FACSort with linear fluorescence amplification. The net mean fluorescence channel number was calculated as the mean fluorescence channel number of cells incubated with immune primary antibody minus that of cells incubated with the non-immune primary reagent.

Activation of Mast Cells. mBMMC (1×10^7/ml) were incubated for 1 h at 4°C in 50% WCM containing mAb B23.1 or RATNP 17.3 (20 μg/ml each), rat IgE (3.25 μ/ml), rat IgE + RATNP, or rat IgE + dilutions of mAb B23.1. After being washed once by centrifugation at 4°C, replicate cell pellets were resuspended on ice at their original volume with 50% WCM either alone or containing 25 μg/ml F(ab')$_2$ mouse IgG anti-rat IgG (heavy and light chain reactive) (Jackson Immunoresearch). The cells were then incubated for 15 min at 37°C with agitation, and 50-μl samples were removed from each tube in duplicate and diluted with an equal volume of 0.15 M EDTA in HBSS. The cells were sedimented by centrifugation at 250 *g* for 5 min at 4°C, the supernatants were decanted and retained, and the pellets were resuspended to their original volumes with a 1:1 (v/v) mixture of 5% WCM/0.15 M EDTA and sonicated on ice.

β-hexosaminidase was quantitated in a 30 μl sample of each supernatant and pellet in duplicate by spectrophotometric analysis of the hydrolysis of p-nitrophenyl-β-D-2-acetamido-2-deoxygluco-pyranoside

(Robinson et al., 1968, Biochem. J. 107:321-327). The percent release values for each experimental condition were calculated by the formula $[S/(S + P)] \times 100$, where S and P are the mediator contents of the samples of each supernatant and cell pellet, respectively. The net percent release values were obtained by subtracting the percent release of sensitized cells incubated in 50% WCM alone from that of replicate cells challenged with $F(ab')_2$ mouse IgG anti-rat IgG heavy and light chains. LTC_4 release was measured by radioimmunoassay (PerSeptive Diagnostics, Cambridge, MA) as described (Katz et al., 1992, J. Immunol. 148:868-871).

RESULTS

Analysis of the Relationship Between the gp49 Family and Other Members of the Ig Superfamily. A homology-based search of the amino acid sequences in the Brookhaven, SWISS-PROT, PIR, translated Genbank, and Kabat databases revealed that both gp49A and gp49B1 possess greatest homology with the human myeloid FcαR, the bovine Fcγ2R, and members of the human KIR family that are expressed on NK cells and certain cytotoxic T lymphocytes. To determine formally whether a relationship exists between gp49B1 and the other receptors, gp49B1 was compared by ALIGN analysis, used previously to define gp49A and gp49B1 as members of the Ig superfamily (Arm et al., 1991, J. Biol. Chem. 266-15966-15973; and Castells et al., 1994, J. Biol. Chem. 269:8393-8401), with receptors of the Ig superfamily that like gp49B1 possess two C2 type, Ig-like domains. Analysis of the complete amino acid sequences revealed statistically significant homology (ALIGN scores ≥ 3) with human FcαR, bovine Fcγ2R, and eight members of the KIR family, but not with mouse and human FcγR species or FcεRIα. Delineation of the two groups of proteins was also observed when each Ig-like domain of gp49, including

20 amino acids flanking each end (William et al., 1988, Annu. Rev. Immunol. 6:381-405), was compared by ALIGN analysis with representative molecules. To assess the relationship of these proteins by another means, evolutionary tree analysis of the full-length amino acid sequences was conducted with the Clustal W program (Thompson et al., 1994, Nucleic Acids Research 22:4673-4680) to perform multiple sequence alignment and PHYLIP Drawtree (Felsenstein, J., 1989, Cladistics 5:164) with default setting to generate the tree. The segregation of the mouse gp49, bovine Fcγ2R, and human FcαR and KIR proteins into a family distinct from the other receptors was confirmed in the analysis because their branches localized as a group that radiated ~180° away from the branches that contained the other receptors analyzed.

Analysis of the Binding Specificity of mAb B23.1 for gp49B1 and gp49A. Stable transfectants that express gp49A or gp49B1 were generated in the mouse P815 mastocytoma cell line, which binds bind little or no mAb B23.1 (LeBlanc et al., 1984, Cell. Immunology 83:242-254). As determined by flow cytometry, mAb B23.1 bound to gp49B1, but not gp49A, transfectants as compared with background binding to P815 cells transfected with the pMH-NEO expression vector alone (Fig. 2). In contrast, anti-gp49A$_{72-87}$ IgG bound to gp49A, but not gp49B1 transfectants, thereby demonstrating that gp49A was expressed on the surface of the appropriate transfectants. Thus, it is reasonable to assume that cell surface gp49B1 can be selectively bound by mAb B23.1 on non-transfected cells, such as mBMMC.

Effects of Ligation of gp49B1 and FcεRI on Mast Cell Activation. The cytoplasmic domain of gp49B1 contains two sequences that conform to the ITIM core consensus (Olcese et al., 1996, J. Immunol. 156:4531-4534), namely, <u>I</u>V<u>Y</u>AQ<u>V</u> and <u>V</u>T<u>Y</u>AQ<u>L</u> (Castells et al., 1994,

J. Biol. Chem. 269:8393-8401). To determine whether coligation of gp49B1 with FcεRI altered activation-secretion, mBMMC were incubated at 4°C with rat IgE (3.25 μg/ml) alone or together with the RATNP 17.3 isotype control (20 μg/ml) or incremental concentrations of mAb B23.1 (0.625 to 20 μg/ml). Samples of cells were also incubated with RATNP 17.3 or mAb B23.1 alone. The cells were washed once and incubated for 15 min at 37°C in medium either alone or with the coligating antibody, a $F(ab')_2$ mouse IgG that is reactive with the light chains of rat IgE and IgM. No release of the secretory granule mediator β-hexosaminidase occurred unless mBMMC were primed with rat IgE in the first step and then stimulated with the second antibody. The release of β-hexosaminidase was inhibited in a dose-dependent fashion when mBMMC were concomitantly incubated with the fixed concentration of rat IgE and incremental concentrations of mAb B23.1 before coligation of their respective FcεRI and gp49B1 molecules. The release of LTC_4 was inhibited in a similar manner.

DISCUSSION

We have established that the coligation of gp49 molecules with FcεRI inhibits mast cell activation-responses. The recognition that gp49 molecules might be inhibitory proteins initially arose from ALIGN analysis of the amino acid sequences of gp49B1 versus other members of the Ig superfamily containing two C2-type, Ig-like domains. This analysis revealed statistically significant homology between gp49B1 and human KIRs. There was also homology between gp49B1, human FcαR, and bovine Fcγ2R, but not with other FcγR species or FcεRI of the human and mouse. The segregation of the homologous proteins into a distinct family was supported by separate evolutionary tree analysis (Thompson et al., 1994, Nucleic Acids Research 22:4673-4680; and Felsenstein, J.,

1989, Cladistics 5:164). A second finding suggested a basis for a function for gp49B1, namely, suppression of an activation event in mast cells, because the cytoplasmic domain possessed two ITIMs, a motif that is also recognized in FcγRIIb species and in KIRs of NK cells and T lymphocyte subsets. Moreover, although the cytoplasmic domain of mouse FcγRIIb1 contains only one ITIM, coligation of this Fc receptor with the B lymphocyte antigen receptor or with FcεRI inhibits B lymphocyte and mast cell activation, respectively (Muta et al., 1994, Nature 368:70-73; D'Ambrisio et al., 1995, Science 268-293-297; and Daëron et al., 1995, J. Clin. Invest. 95:577-585). Of note is the finding that mouse gp49B1 does not exhibit statistically significant amino acid sequence homology with mouse FcγRIIb1. Thus, our findings establish that separate homology-defined families within the Ig superfamily, with possibly distinct counterligands, inhibit mast cell activation.

We previously demonstrated that COS cells transfected with a cDNA encoding gp49B1 are immunoreactive with mAb B23.1 (Castells et al., 1994, J. Biol. Chem. 269:8393-8401). However, the specificity of the mAb with regard to gp49A was unknown because of the 89% identity between the extracellular domains of gp49B1 and gp49A. To address this issue, stable transfectants expressing either the gp49A or the gp49B1 cDNA were created in the P815 mastocytoma cell line, which binds little or no mAb B23.1. In addition, to measure specifically the expression of gp49A in transfectants, rabbit anti-gp49A$_{72-87}$ IgG was prepared to a peptide in the extracellular domain that differs in gp49A and gp49B1 (Castells et al., 1994, J. Biol. Chem. 269:8393-8401). mAb B23.1 specifically bound to transfectants that expressed gp49B1, and anti-gp49$_{72-87}$ Ig specifically bound to gp49A transfectants. Because mAb B23.1 recognizes cell

surface gp49B1, but not gp49B1 after exposure to SDS, mAb B23.1 appears to recognize a conformation-dependent, gp49B1-specific epitope within an extracellular domain that is 89% identical to that of gp49A. The latter finding is reminiscent of the fact that certain anti-human KIR mAbs specifically recognize individual receptor species, even though their extracellular domains differ only by several scattered amino acids (Wagtmannet al., 1995, Immunity 2:439-449).

With use of BMMC primed with rat IgM mAb B23.1 anti-gp49B1 and rat IgE, the effects of coligation of gp49B1 and FcεRI were established with a second antibody, namely, $F(ab')_2$ mouse IgG anti-rat IgG. This antibody recognized the light chains of both rat mAb B23.1 and rat IgE, as assessed by flow cytometry. Neither mAb B23.1 nor the isotype-matched mAb RATNP 17.3 alone elicited the release of mediators from mBMMC after exposure to second antibody. However, when mAb B23.1 and rat IgE on the surface of the mast cells were coligated with the second antibody, a mAb B23.1-dependent, dose-related inhibition of β-hexosaminidase and LTC_4 release occurred. This inhibition was not the result of a limitation in the amount of $F(ab')_2$ mouse IgG anti-rat IgG available to react with FcεRI, because both the rat IgE and mAb B23.1 were added to the cells at concentrations that were not saturating for the binding of second antibody, as determined by flow cytometry. The finding that gp49B1 contains two core ITIM motifs and suppresses secretory granule and lipid-derived mediator release when coligated with FcεRI leads us to propose that gp49B1 be designated "mouse mast cell inhibitory receptor" (mMIR).

The mechanism by which ITIMs inhibit cellular activation events appears to be through the downstream recruitment of one or more tyrosine phosphatases that reverse a tyrosine phosphorylation step(s) critical to

progressive signal transduction. Coligation of the B lymphocyte antigen receptor with FcγRIIb1 causes tyrosine phosphorylation of each receptor with different resultant intracellular protein associations. Whereas the B lymphocyte antigen receptor associates with *syk* kinase via the receptor's immunoreceptor tyrosine-containing activation motif in the Ig-α and Ig-β subunits (Muta et al.), phosphopeptides encompassing the FcγRIIB1 ITIM bind SHP-1 (D'Ambrosio et al.). Furthermore, tyrosine-phosphorylated KIR peptides from the two ITIMs of certain human KIRs bind SHP-1 and SHP-2 (formerly PTP1D) (Burshytn et al., 1996, Immunity 4:77-85; and Olcese et al., 1996, J. Immunol. 156:4531-4534). Thus, ITIMs may mediate inhibitory effects by recruiting tyrosine phosphatases that could reverse and/or suppress tyrosine phosphorylation events at one or more steps of signal transduction cascades that activate cellular functions.

The finding that mMIR is homologous to members of the Ig superfamily that bind other members of the superfamily (e.g., Fc receptors/Ig, KIRs/MHC class I) suggests that its counterligand may also be a member of the Ig superfamily. Although mouse NK cells express a family of KIRs (Ly49) that contain the ITIM motif (Olcese et al., 1996, J. Immunol. 156:4531-4534) and function like human NK cell KIRs in interacting with MHC class I molecules, the Ly49 family consists of type II transmembrane proteins with an extracellular C-type lectin domain rather than an Ig-like structure (Yokoyama et al., 1995, Seminars in Immunology 7:89-101). It is possible therefore, based on the structural and functional considerations presented here, that mMIR may belong to a family of molecules that are the mouse equivalents of human NK and T lymphocyte KIRs.

Comparison by ALIGN analysis of the predicted amino acid sequence of gp49A with selected receptors of

the Ig superfamily containing two C2-type Ig-like domains was accomplished as follows. ALIGN scores greater than three, as depicted by the vertical dashed line, indicate that the similarities between those molecules and gp49A are significantly greater than would occur by chance alone. Data are expressed as mean ± SEM of three ALIGN analyses for each pairing. Sequences and nomenclature of NKATs 1-4 and KIR clones 39, 42, 43, and 49 are from the following references respectively, Colonna et al., 1995, Science 268:405-408 and Wagtmann et al., 1995, Immunity 2:439-449.

Flow cytometric analysis of the binding of rat IgM mAb B23.1 (top) and rabbit IgG anti-gp49A_{72-87} IgG (bottom) to pMH-NEO vector control, gp49A, and gp49B1 transfectants was performed as follows. Cells were incubated with primary antibodies for 30 min at 4°C, washed, and incubated with $F(ab')_2$ fragments of appropriate, FITC-labeled secondary antibodies for 30 min at 4°C before flow cytometry. Data are expressed as net mean fluorescence channel numbers ± SEM, n=5 (anti-gp49A_{72-87} IgG) and n=3 (mAb B23.1).

The following was used for dose-dependent analysis of the effects of increasing concentrations of mAb B23.1 on the response of mBMMC to coligation of gp49B1 and FcεRI as assessed by IgE activation-induced release of β-hexosaminidase and LTC_4. Samples of cells were incubated with the indicated concentrations of antibody for 1 h at 4°C, washed, and incubated with $F(ab')_2$ mouse IgG anti-rat heavy and light chain IgG for 15 min at 37°C. β-hexosaminidase and LTC_4 were measured by spectrophotometric assay and radioimmunoassay, respectively. β-hexosaminidase data are expressed as mean ± SEM, n=4; LTC_4 data are expressed as mean ± half-range, n=2. The net % β-hexosaminidase release and LTC_4 release when control rat IgM mAb RATNP 17.3 (20 μg/ml)

was combined with the rat IgE sensitization step before coligation and were not appreciably different from the mediator release from cells incubated with rat IgE alone. The net % β-hexosaminidase release when cells were exposed to RATNP 17.3 alone and then to either medium or $F(ab')_2$ mouse IgG anti-rat IgG in the second step was 8 ± 4% and 3 ± 1% (n=3); and for cells that were exposed to mAb B23.1 alone (20 μg/ml) in the first step were 7 ± 3% and 4 ± 2%, respectively (n=4). Release of LTC_4 under these conditions was undetectable.

When B lymphocytes are exposed to $F(ab')_2$ fragments of anti-membrane Ig antibody, a signal transduction cascade is elicited through the B lymphocyte antigen receptor that results in B lymphocyte proliferation and differentiation into antibody-secreting cells (Cambier et al., 1987, Ann. Rev. Immunol. 5:175-199 and Amigorena et al., 1992, Science 256:1808-1812). In contrast, stimulation with intact anti-membrane Ig results in attenuated B lymphocyte signal transduction, as measured by the influx of extracellular calcium (Wilson et al., 1987, J. Immunol. 138:1712-1718) and levels of cellular inositol trisphosphates (Bijsterbosch et al., 1985, J. Exp. Med. 162:1825-1836). The fact that the suppression of B cell stimulation can be prevented by blocking the binding of intact anti-membrane Ig to Fc receptors for IgG, type IIb1 (FcγRIIb1) on the B lymphocyte (4,8), indicates that coligation of the B lymphocyte antigen receptor with FcγRIIb1 inhibits B lymphocyte stimulation. Amino acid substitution experiments revealed that a 13-amino acid region of the cytoplasmic domain of FcγRIIb1 is sufficient to inhibit B lymphocyte stimulation (Muta et al., 1994, Nature 368:70-73). Furthermore, a tyrosine in the 13-amino acid sequence is phosphorylated on coligation of the two receptors, and the mutation of this tyrosine to phenylalanine prevents the FcγRIIb1-mediated

inhibition when the two receptors undergo coligation (Muta et al., 1994, Nature 368:70-73). The cytosolic tyrosine phosphatase SHP-1 (formerly termed protein tyrosine phosphatase [PTP] 1C and hematopoietic cell phosphatase) coimmunoprecipitates with FcγRIIb1 only when the receptor is phosphorylated on tyrosine after coligation. As assessed by SDS-PAGE immunoblots, a phosphorylated, but not non-phosphorylated, peptide matching this region of FcγRIIb1 binds SHP-1 from B lymphocyte lysates in equal quantities whether the cells are unstimulated, stimulated, or stimulation-inhibited (D'Ambrosio et al., 1995, Science 268:293-297); thus, phosphorylation of the FcγRIIb1 motif is critical to the binding of SHP-1. Moreover, because binding of the phosphorylated, but not non-phosphorylated, peptide increases the activity of SHP-1 several-fold, phosphorylated FcγRIIb1 may both recruit and regulate the activity of SHP-1. Because signal transduction leading to the activation of B lymphocytes requires tyrosine kinase phosphorylation of multiple substrates, coligation of FcγRIIb1 with the B lymphocyte antigen receptor may promote the intracellular dephosphorylation of one or more substrates critical to activation signaling.

The coligation of FcγRIIb1 or the alternative splicing variant FcγRIIb2 with FcεRI on mouse bone marrow-derived mast cells (mBMMC) results in the downregulation of exocytosis elicited through FcεRI (Daëron et al., 1995, J. Clin. Invest. 95:577-585). In a rat mast cell line transfected with mouse FcγRIIb2, coligation of the mouse receptor with endogenous rat FcεRI inhibited exocytosis and the secretion of tumor necrosis factor-α (Daëron et al., 1995, J. Clin. Invest. 95:577-585). Simultaneous, independent ligation of FcγRIIb2 and FcεRI did not inhibit exocytosis in the transfectants, nor did coligation when the cytoplasmic domain of the FcγRII

species was deleted. Thus, the apposition of cytoplasmic domain sequences in FcγRIIb species with FcεRI downregulates mast cell activation.

Human natural killer (NK) cells and T lymphocyte subsets express a group of Ig superfamily cell surface receptors, termed "killer cell inhibitory receptors" (KIRs) that recognize allelic groups of the major histocompatibility complex (MHC) class I molecules (Moretta et al., 1996, Annu. Rev. Immunol. 14:619-648; Lanier et al., 1996, Immunol Today 17:86-91; Moretta et al., 1994, Advances in Immunology 55:341-380; Yokoyama et al., 1995, Seminars in Immunology 7:89-101; and Gumperz et al., 1995, Nature 378:245-248). The engagement of KIRs by the appropriate MHC class I molecules inhibits the activation pathways of both NK cells and T lymphocytes for cytotoxic effector function (Moretta et al., 1994, Advances in Immunology 55:341-380; Yokoyama et al., 1995, Seminars in Immunology 7:89-101; Phillips et al., 1995, Science 268:403-405; and Mingari et al., 1995, Int. Immunol. 7:697-703); and this recognition mechanism protects normal, autologous cells. Both the KIRs and the FcγRIIb species contain a core consensus motif in their cytoplasmic domains, I/V x Y x x L/V, termed the immunoreceptor tyrosine-based inhibition motif (ITIM) (Yokoyama et al., 1995, Seminars in Immunology 7:89-101; and Raulet et al., 1995, Seminars in Immunology 7:103-107); the ITIM core is within the 13-amino acid region of FcγRIIb species that mediates inhibition of B lymphocyte stimulation. Phosphorylated, but not non-phosphorylated, peptides containing ITIMs from KIR cytoplasmic domains interact with and stimulate SHP-1 activity (Burshtyn et al., 1996, Immunity 4:77-85; and Olcese et al., 1996, J. Immunol. 156:4531-4534). Moreover, the transfection of cytolytic, KIR-deficient NK cells exhibiting low levels of SHP-1 with constructs encoding a KIR and active SHP-1

inhibits their cytotoxicity for an appropriate target cell, whereas cotransfection with a construct encoding the KIR and enzymatically inactive SHP-1 does not. Thus, the recruitment of SHP-1 activity is directly involved in KIR-mediated inhibition of NK cell cytotoxicity (Burshtyn et al., 1996, Immunity 4:77-85). The identity of the activating receptor(s) on NK cells whose cytotoxic function signals are inhibited by SHP-1 is unknown.

The amino acid sequence of gp49B1 bears statistically significant homologies to human KIRs and to two Fc receptors, human Fc receptors for IgA (FcαR) and bovine Fc receptors for IgG2 (Fcγ2R), that together form a newly recognized family within the Ig superfamily. This family does not include FcγRIIb or the other FcγR of mouse or human origin. We also show that the cytoplasmic domain of gp49B1 contains two ITIMs, implying functional homology with members of the Ig superfamily that have amino acid sequences that are not homologous to that of gp49B1. Using stable transfectants that express either gp49B1 or gp49A, we demonstrate that mAb B23.1 specifically binds gp49B1 on the surface of cells. Most importantly, we establish that the coligation of FcϵRI and gp49B1 on the surface of mast cells suppresses FcϵRI-mediated exocytosis, defined by release of the secretory granule mediator β-hexosaminidase, and the generation of the membrane derived pro-inflammatory lipid mediator leukotriene (LT) C_4. Thus, the apposition of gp49B1 with FcϵRI sends a negative regulatory signal that inhibits the signal transduction cascade emanating from FcϵRI, thereby attenuating mast cell activation.

By ALIGN comparison of the amino acid sequence of gp49's with numerous receptors of the Ig superfamily, a newly recognized family has been established that includes gp49, the human myeloid cell Fc receptor for IgA, the bovine myeloid cell Fc receptor for IgG2, and

the human killer cell inhibitory receptors (KIRs) expressed on natural killer (NK) cells and T lymphocyte subsets. Furthermore, the cytoplasmic domain of gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs (ITIMs) that are also present in KIRs; these motifs downregulate NK cell and T cell activation signals that lead to cytotoxic activity. As assessed by flow cytometry with transfectants that express either gp49B1 or gp49A, which are 89% identical in the amino acid sequences of their extracellular domains, mAb B23.1 was shown to recognize only gp49B1. Coligation of mAb B23.1 bound to gp49B1 and IgE fixed to the high affinity Fc receptor for IgE (FcϵRI) on the surface of mouse bone marrow-derived mast cells inhibited exocytosis in a dose-related manner, as defined by the release of the secretory granule constituent β-hexosaminidase, as well as the generation of the membrane-derived lipid mediator, leukotriene C4. Thus, gp49B1 is an endogenous, ITIM-containing integral cell surface protein that downregulates FcϵRI-mediated release of proinflammatory mediators from mast cells. Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described herein. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting

What is claimed is:

1. A substantially pure human gp49 polypeptide.

2. The polypeptide of claim 1 wherein said polypeptide is soluble in physiological fluid.

3. The polypeptide of claim 1 wherein said polypeptide comprises at least one of the following functional human gp49 domains:

a) a human gp49 signal peptide domain;

b) a human gp49 C-2 Ig domain;

c) a human gp49 transmembrane domain;

d) a human gp49 cytoplasmic tail.

4. The polypeptide of claim 3 in which the polypeptide comprises an ITIM motif of a human gp49 cytoplasmic tail.

5. The polypeptide of claim 3 in which the functional human gp49 domain comprises a sequence of at least residues that is identical to a sequence of HM18 or of HM43.

6. A substantially pure fusion polypeptide comprising a human gp49 component and FcεRI.

7. A substantially pure fusion polypeptide comprising a human gp49 component and a detectable marker.

8. A recombinant nucleic acid encoding a human gp49 polypeptide according to claim 1.

9. A recombinant nucleic acid encoding the fusion polypeptide of claim 6,

10. A cell comprising the recombinant nucleic acid of claim 8 or claim 9.

11. A vector comprising the recombinant nucleic acid of claim 8 or claim 9.

12. An antibody which selectively binds to a gp 49 polypeptide according to claim 1.

13. A pharmaceutical composition comprising the polypeptide of claim 1 or the nucleic acid of claim 8 or claim 9.

14. A method of treating an undesired immune response in a patient by administering a mammalian gp49 polypeptide or nucleic acid encoding a mammalian gp49 polypeptide.

15. A method or treating a mast cell-related disease by administering a mammalian gp49 polypeptide or nucleic acid encoding a mammalian gp49 polypeptide.

16. The method of claim 14 or claim 15 wherein the gp49 polypeptide is a human gp49 polypeptide.

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-45   -40        -30        -20        -10
TGAGA TGAGAGCTGC CGACAGTTGG GGGTCAAGGG AGGAGACGCC ATG ATC CCC ACC
                                                  Met Ile Pro Thr

15              30              45              60
TTC ACG GCT CTG CTC TGC CTC GGG CTG AGT CTG GGC CCC AGG ACC CAC ATG CAG
Phe Thr Ala Leu Leu Cys Leu Gly Leu Ser Leu Gly Pro Arg Thr His Met Gln

        75              90              105             120
GCA GGG CCC CTC CCC AAA CCC ACC CTC TGG GCT GAG CCA GGC TCT GTG ATC AGC
Ala Gly Pro Leu Pro Lys Pro Thr Leu Trp Ala Glu Pro Gly Ser Val Ile Ser

                135             150             165
TGG GGG AAC TCT GTG ACC ATC TGG TGT CAG GGG ACC CTG GAG GCT CGG GAG TAC
Trp Gly Asn Ser Val Thr Ile Trp Cys Gln Gly Thr Leu Glu Ala Arg Glu Tyr

    180             195             210             225
CGT CTG GAT AAA GAG GAA AGC CCA GCA CCC TGG GAC AGA CAG AAC CCA CTG GAG
Arg Leu Asp Lys Glu Glu Ser Pro Ala Pro Trp Asp Arg Gln Asn Pro Leu Glu

            240             255             270
CCC AAG AAC AAG GCC AGA TTC TCC ATC CCA TCC ATG ACA GAG GAC TAT GCA GGG
Pro Lys Asn Lys Ala Arg Phe Ser Ile Pro Ser Met Thr Glu Asp Tyr Ala Gly

285             300             315             330
AGA TAC CGC TGT TAC TAT CGC AGC CCT GTA GGC TGG TCA CAG CCC AGT GAC CCC
Arg Tyr Arg Cys Tyr Tyr Arg Ser Pro Val Gly Trp Ser Gln Pro Ser Asp Pro

        345             360             375             390
CTG GAG CTG GTG ATG ACA GGA GCC TAC AGT AAA CCC ACC CTT TCA GCC CTG CCG
Leu Glu Leu Val Met Thr Gly Ala Tyr Ser Lys Pro Thr Leu Ser Ala Leu Pro

                405             420             435
AGT CCT CTT GTG ACC TCA GGA AAG AGC GTG ACC CTG CTG TGT CAG TCA CGG AGC
Ser Pro Leu Val Thr Ser Gly Lys Ser Val Thr Leu Leu Cys Gln Ser Arg Ser

    450             465             480             495
CCA ATG GAC ACT TTC CTT CTG ATC AAG GAG CGG GCA GCC CAT CCC CTA CTG CAT
Pro Met Asp Thr Phe Leu Leu Ile Lys Glu Arg Ala Ala His Pro Leu Leu His

            510             525             540
CTG AGA TCA GAG CAC GGA GCT CAG CAG CAC CAG GCT GAA TTC CCC ATG AGT CCT
Leu Arg Ser Glu His Gly Ala Gln Gln His Gln Ala Glu Phe Pro Met Ser Pro

555             570             585             600
GTG ACC TCA GTG CAC GGG GGG ACC TAC AGG TGC TTC AGC TCA CAC GGC TTC TCC
Val Thr Ser Val His Gly Gly Thr Tyr Arg Cys Phe Ser Ser His Gly Phe Ser

        615             630             645             660
CAC TAC CTG CTG TCA CAC CCC AGT GAC CCC CTG GAG CTC ATA GTC TCA GGA TCC
His Tyr Leu Leu Ser His Pro Ser Asp Pro Leu Glu Leu Ile Val Ser Gly Ser

                675             690             705
TTG GAG GGT CCC AGG CCC TCA CCC ACA AGG TCC GTC TCA ACA GCT GCA GGC CCT
Leu Glu Gly Pro Arg Pro Ser Pr  Thr Arg Ser Val Ser Thr Ala Ala Gly Pro

    720             735             750             765
GAG GAC CAG CCC CTC ATG CCT ACA GGG TCA GTC CCC CAC AGT GGT CTG AGA AGG
Glu Asp Gln Pro Leu Met Pro Thr Gly Ser Val Pr  His Ser Gly Leu Arg Arg
```

FIG. 1A

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780 795 810
CAC TGG GAG GTA CTG ATC GGG GTC TTG GTG GTC TCC ATC CTG CTT CTC TCC CTC
His Trp Glu Val Leu Ile Gly Val Leu Val Val Ser Ile Leu Leu Leu Ser Leu

825 840 855 870
CTC CTC TTC CTC CTC CTC CAA CAC TGG CGT CAG GGA AAA CAC AGG ACA TTG GCC
Leu Leu Phe Leu Leu Leu Gln His Trp Arg Gln Gly Lys His Arg Thr Leu Ala

885 900 915 930
CAG AGA CAG GCT GAT TTC CAA CGT CCT CCA GGG GCT GCC GAG CCA GAG CCC AAG
Gln Arg Gln Ala Asp Phe Gln Arg Pro Pro Gly Ala Ala Glu Pro Glu Pro Lys

945 960 975
GAC GGG GGC CTA CAG AGG AGG TCC AGC CCA GCT GCT GAC GTC CAG GGA GAA AAC
Asp Gly Gly Leu Gln Arg Arg Ser Ser Pro Ala Ala Asp Val Gln Gly Glu Asn

990 1005 1020 1035
TTC TGT GCT GCC GTG AAG AAC ACA CAG CCT GAG GAC GGG GTG GAA ATG GAC ACT
Phe Cys Ala Ala Val Lys Asn Thr Gln Pro Glu Asp Gly Val Glu Met Asp Thr

1050 1065 1080
CGG CAG AGC CCA CAC GAT GAA GAC CCC CAG GCA GTG ACG TAT GCC AAG GTG AAA
Arg Gln Ser Pro His Asp Glu Asp Pro Gln Ala Val Thr Tyr Ala Lys Val Lys

1095 1110 1125 1140
CAC TCC AGA CCT AGG AGA GAA ATG GCC TCT CCT CCC TCC CCA CTG TCT GGG GAA
His Ser Arg Pro Arg Arg Glu Met Ala Ser Pro Pro Ser Pro Leu Ser Gly Glu

1155 1170 1185 1200
TTC CTG GAC ACA AAG GAC AGA CAG GCA GAA GAG GAC AGA CAG ATG GAC ACT GAG
Phe Leu Asp Thr Lys Asp Arg Gln Ala Glu Glu Asp Arg Gln Met Asp Thr Glu

1215 1230 1245
GCT GCT GCA TCT GAA GCC CCC CAG GAT GTG ACC TAC GCC CAG CTG CAC AGC TTT
Ala Ala Ala Ser Glu Ala Pro Gln Asp Val Thr Tyr Ala Gln Leu His Ser Phe

1260 1275 1290 1305
ACC CTC AGA CAG AAG GCA ACT GAG CCT CCT CCA TCC CAG GAA GGG GCC TCT CCA
Thr Leu Arg Gln Lys Ala Thr Glu Pro Pro Pro Ser Gln Glu Gly Ala Ser Pro

1320 1335 1,360
GCT GAG CCC AGT GTC TAT GCC ACT CTG GCC ATC CAC TAA TCC AGGGGGGACC
Ala Glu Pro Ser Val Tyr Ala Thr Leu Ala Ile His *

1,370 1,380 1,390 1,400 1,410 1,420
CAGACCCCAC AAGCCATGGA GACTCAGGAC CCCAGAAGGC ATGGAAGCTG CCTCCAGTAG

1,430 1,440 1,450 1,460 1,470 1,480
ACATCACTGA ACCCCAGCCA GCCCAGACCC CTGACACAGA CCACTAGAAG ATTCCGGGAA

1,490 1,500 1,510 1,520 1,530 1,540
CGTTGGGAGT CACCTGATTC TGCAAAGATA AATAATATCC CTGCATTATC AAAATAAAGT

1,550 1,560 1,570 1,580
AGCAGACCTC TCAATTCAAA AAAAAAAAAA AACGGAATTC

FIG. 1B

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```
   -60        -50        -40        -30        -20
CT CTGTCCTGCC AGCACCGAGG GCTCATCCAT CCACAGAGCA GTGCAGTGGG

-10                             15                      30          45
AGGAGACGCC  ATG ACC TCC ATC CTC ACG GTC CTG ATC TGT CTC GGG CTG AGC CTG
            Met Thr Ser Ile Leu Thr Val Leu Ile Cys Leu Gly Leu Ser Leu

                60          75          90
GAC CCC AGG ACC CAC GTG CAG GCA GGG CCC CTC CCC AAG CCC ACC CTC TGG GCT
Asp Pro Arg Thr His Val Gln Ala Gly Pro Leu Pro Lys Pro Thr Leu Trp Ala

    105         120         135         150
GAG CCA GGC TCT GTG ATC ACC CAA GGG AGT CCT GTG ACC CTC AGG TGT CAG GGG
Glu Pro Gly Ser Val Ile Thr Gln Gly Ser Pro Val Thr Leu Arg Cys Gln Gly

        165         180         195
AGC CTG GAG ACG CAG GAG TAC CAT CTA TAT AGA GAA AAG AAA ACA GCA CTC TGG
Ser Leu Glu Thr Gln Glu Tyr His Leu Tyr Arg Glu Lys Lys Thr Ala Leu Trp

210             225         240         255
ATT ACA CGG ATC CCA CAG GAG CTT GTG AAG AAG GGC CAG TTC CCC ATC CTA TCC
Ile Thr Arg Ile Pro Gln Glu Leu Val Lys Lys Gly Gln Phe Pro Ile Leu Ser

        270         285         300         315
ATC ACC TGG GAA CAT GCA GGG CGG TAT TGC TGT ATC TAT GGC AGC CAC ACT GCA
Ile Thr Trp Glu His Ala Gly Arg Tyr Cys Cys Ile Tyr Gly Ser His Thr Ala

            330         345         360
GGC CTC TCA GAG AGC AGT GAC CCC CTG GAG CTG GTG GTG ACA GGA GCC TAC AGC
Gly Leu Ser Glu Ser Ser Asp Pro Leu Glu Leu Val Val Thr Gly Ala Tyr Ser

    375         390         405         420
AAA CCC ACC CTC TCA GCT CTG CCC AGC CCT GTG GTG ACC TCA GGA GGG AAT GTG
Lys Pro Thr Leu Ser Ala Leu Pro Ser Pro Val Val Thr Ser Gly Gly Asn Val

        435         450         465
ACC ATC CAG TGT GAC TCA CAG GTG GCA TTT GAT GGC TTC ATT CTG TGT AAG GAA
Thr Ile Gln Cys Asp Ser Gln Val Ala Phe Asp Gly Phe Ile Leu Cys Lys Glu

480             495         510         525
GGA GAA GAT GAA CAC CCA CAA TGC CTG AAC TCC CAT TCC CAT GCC CGT GGG TCA
Gly Glu Asp Glu His Pro Gln Cys Leu Asn Ser His Ser His Ala Arg Gly Ser

        540         555         570         585
TCC CGG GCC ATC TTC TCC GTG GGC CCC GTG AGC CCA AGT CGC AGG TGG TCG TAC
Ser Arg Ala Ile Phe Ser Val Gly Pro Val Ser Pro Ser Arg Arg Trp Ser Tyr

                600         615         630
AGG TGC TAT GGT TAT GAC TCG CGC GCT CCC TAT GTG TGG TCT CTA CCC AGT GAT
Arg Cys Tyr Gly Tyr Asp Ser Arg Ala Pro Tyr Val Trp Ser Leu Pro Ser Asp

    645         660         675         690
CTC CTG GGG CTC CTG GTC CCA GGT GTT TCT AAG AAG CCA TCA CTC TCA GTG CAG
Leu Leu Gly Leu Leu Val Pro Gly Val Ser Lys Lys Pro Ser Leu Ser Val Gln

        705         720         735
CCG GGT CCT GTC GTG GCC CCT GGG GAG AA  CTG ACC TTC CAG TGT GGC TCT GAT
Pro Gly Pro Val Val Ala Pro Gly Glu Lys Leu Thr Phe Gln Cys Gly Ser Asp
```

FIG. 2A

```
750                 765                 780                 795
GCC GGC TAC GAC AGA TTT GTT CTG TAC AAG GAG TGG GGA CGT GAC TTC CTC CAG
Ala Gly Tyr Asp Arg Phe Val Leu Tyr Lys Glu Trp Gly Arg Asp Phe Leu Gln

    810                 825                 840                 855
CGC CCT GGC CGG CAG CCC CAG GCT GGG CTC TCC CAG GCC AAC TTC ACC CTG GGC
Arg Pro Gly Arg Gln Pro Gln Ala Gly Leu Ser Gln Ala Asn Phe Thr Leu Gly

                870                 885                 900
CCT GTG AGC CGC TCC TAC GGG GGC CAG TAC ACA TGC TCC GGT GCA TAC AAC CTC
Pro Val Ser Arg Ser Tyr Gly Gly Gln Tyr Thr Cys Ser Gly Ala Tyr Asn Leu

    915                 930                 945                 960
TCC TCC GAG TGG TCG GCC CCC AGC GAC CCC CTG GAC ATC CTG ATC ACA GGA CAG
Ser Ser Glu Trp Ser Ala Pro Ser Asp Pro Leu Asp Ile Leu Ile Thr Gly Gln

            975                 990                 1005
ATC CGT GCC AGA CCC TTC CTC TCC GTG CGG CCG GGC CCC ACA GTG GCC TCA GGA
Ile Arg Ala Arg Pro Phe Leu Ser Val Arg Pro Gly Pro Thr Val Ala Ser Gly

1020                1035                1050                1065
GAG AAC GTG ACC CTG CTG TGT CAG TCA CAG GGA GGG ATG CAC ACT TTC CTT TTG
Glu Asn Val Thr Leu Leu Cys Gln Ser Gln Gly Gly Met His Thr Phe Leu Leu

    1080                1095                1110                1125
ACC AAG GAG GGG GCA GCT GAT TCC CCG CTG CGT CTA AAA TCA AAG CGC CAA TCT
Thr Lys Glu Gly Ala Ala Asp Ser Pro Leu Arg Leu Lys Ser Lys Arg Gln Ser

                1140                1155                1170
CAT AAG TAC CAG GCT GAA TTC CCC ATG AGT CCT GTG ACC TCG GCC CAC GCG GGG
His Lys Tyr Gln Ala Glu Phe Pro Met Ser Pro Val Thr Ser Ala His Ala Gly

    1185                1200                1215                1230
ACC TAC AGG TGC TAC GGC TCA CTC AGC TCC AAC CCC TAC CTG CTG ACT CAC CCC
Thr Tyr Arg Cys Tyr Gly Ser Leu Ser Ser Asn Pro Tyr Leu Leu Thr His Pro

            1245                1260                1275
AGT GAC CCC CTG GAG CTC GTG GTC TCA GGA GCA GCT GAG ACC CTC AGC CCA CCA
Ser Asp Pro Leu Glu Leu Val Val Ser Gly Ala Ala Glu Thr Leu Ser Pro Pro

1290                1305                1320    1,330        1,340
CAA AAC AAG TCC GAC TCC AAG GCT GGT GAG TGA GGAGATGCTT  GCCGTGATGA
Gln Asn Lys Ser Asp Ser Lys Ala Gly Glu  *

     1,350      1,360      1,370      1,380      1,390      1,400
CGCTGGGCAC AGAGGGTCAG GTCCTGTCAA GAGGAGCTGG GTGTCCTGGG TGGACATTTG

     1,410      1,420      1,430      1,440      1,450      1,460
AAGAATTATA TTCATTCCAA CTTGAAGAAT TATTCAACAC CTTTAACAAT GTATATGTGA

     1,470      1,480      1,490      1,500      1,510      1,520
AGTACTTTAT TCTTTCATAT TTTAAAAATA AAAGATAATT ATCCATGAAA AAAAAAAAAA

     1,530
AAACGGAATT C
```

FIG. 2B

```
                       -HUMAN
COMPARING:   HM18
             gp49B1 -MOUSE
                                                         FIRST C-2 Ig DOMAIN
          SIGNAL PEPTIDE
MIPTFTALLCLGLSLGPRTHMQAGPLPKPTLWAEPGSVISWGNSVTIWCQGTLEAREYRLDKEESPAPWD     70
--AML-V--Y---I-E---AV---H----II--------AAYT--IT----SW--QY-H-Y--K-VN---     70

RQNPLEPKNKARFSIPSMTEDYAGRYRCYYRSPVGWSQPSDPLELVMTGAYSKPTLSALPSPLVTSGKSV    140
T-V---TR---K-N-----TS---I-K---E-AA-F-EH--AM---------EN-S--VY--SN----V-I    140

   SECOND C-2 Ig DOMAIN
TLLCQSRSPMDTFLLIKERAAHPLLHLRSEHGAQQ HQAEFPMSPVTSVHGGTYRCFSSHGFSHYLLSHP    210
SFS-S-SIVFGR-I--Q-GKHGLSWT-D-Q-Q-N-PSY-T-VLDA--PN-N--F--YGYFRNEPQVW-K-    210

                                                  TRANSMEMBRANE DOMAIN
SDPLELIVSGSLEGPRPSPTRSVSTAAGPEDQPLMPTGSVPHSGLRRHWEVLIGVLVVSILLLSLLLFLL    280
                                                 --ETYQKI------SFL--FF-----I    280
-NS-D-MI-ETKDQSSTPTED

          CYTOPLASMIC TAIL
LQHWRQGKHRTLAQRQADFQRPPGAAEPEPKDGGLQRRSSPAADVQGENFCAAVKNTQPEDGVEMDTRQS    350
                                                 AN-S-----S-NNA-LNS WN    350
-IGYQY-HKKK
                                                            ITIM
         ITIM
PHDEDPQAVTYAKVKHSRPRREMASPPSPLSGEFLDTKDRQAEEDRQMDTEAAASEAPQDVTYAQLHSFT    420
                                                 T-CKET---------CIR-    420
-QN----GIV--Q--P--LQKD

                                                                         490
LRQKATEPPPSQEGASPAEPSVYATLAIH                                            490
QE-NNS
```

FIG. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15586

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 38/00; C07K 14/705
US CL : 424/185.1; 435/69.1, 320.1, 325; 530/395; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/185.1; 435/69.1, 320.1, 325; 530/395; 536/23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and DIALOG (file=biochem) databases. Key words: gp49? and (protein or glycoprotein)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	ARM, J. P. et al. Molecular cloning of gp49, a cell-surface antigen that is preferentially expressed by mouse mast cell progenitors and is a new member of the immunoglobulin superfamily. J. Biol. Chem. 25 August 1991, Vol. 266, No. 24, pages 15966-15973, see entire document.	12 ----- 1-16
X ---- Y	CASTELLS, M.C. et al. Cloning of the gp49B gene of the immunoglobulin superfamily and demonstration that one of its two products is an early-expressed mast cell surface protein originally described as gp49. J. Biol. Chem. 18 March 1994, Vol. 269, No. 11, pages 8393-8401, see entire document.	12 ----- 1-16

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
19 NOVEMBER 1997	22 DEC 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Thomas Cunningham JAB for
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/15586

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	KATZ, H. R. et al. Activation- and phorbol ester-stimulated phosphorylation of a plasma membrane glycoprotein antigen expressed on mouse IL-3-dependent mast cells and serosal mast cells. J. Immunol. 01 February 1989, Vol. 142, No. 3, pages 919-926, see entire document.	12 ----- 1-16